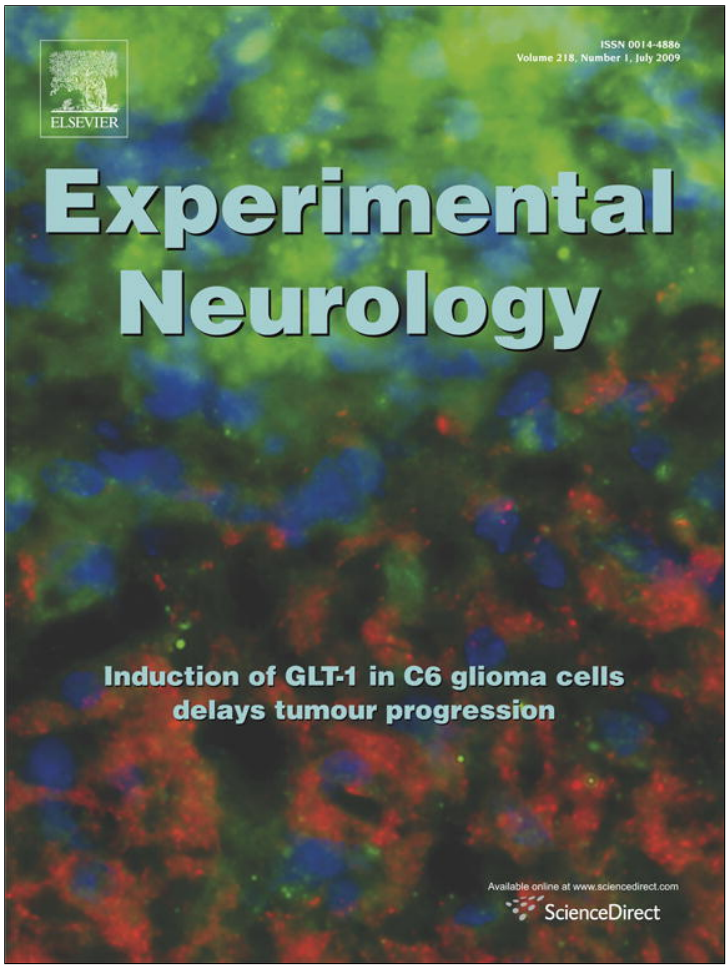

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Enhanced expression of the high affinity glutamate transporter GLT-1 in C6 glioma cells delays tumour progression in rat

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ABSTRACT

High grade gliomas are known to release excitotoxic concentrations of glutamate, a process thought to contribute to their malignant phenotype through enhanced autocrine stimulation of their proliferation and destruction of the surrounding nervous tissue. A model of C6 glioma cells in which expression of the high affinity glutamate transporter GLT-1 can be manipulated both in vivo and in vitro was used in order to investigate the consequences of increasing glutamate clearance on tumour progression. These cells were grafted in the striatum of Wistar rats and doxycycline was administered after validation of tumour development by magnetic resonance imaging. Both GLT-1 expression examined by immunohistochemistry and glutamate transport activity measured on synaptosomes appeared robustly increased in samples from doxycycline-treated animals. Moreover, these rats showed extended survival times as compared to vehicle-treated animals, an effect that was consistent with volumetric data revealing delayed tumour growth. As constitutive deficiency in glutamate clearance at the vicinity of brain tumours is well established, these data illustrate the potential benefit that could be obtained by enhancing glutamate transport by glioma cells in order to reduce their invasive behaviour.

Introduction

Impaired functioning of glutamate transporters results in elevated extracellular glutamate concentration, a common feature of several nervous disorders, including motor neuron disease and Alzheimer's disease (Honig et al., 2000; Munch et al., 2001). Indeed, excessive glutamatergic stimulation of neurons causes excitotoxicity which is physiologically prevented by an active clearance process ensured by astroglial cells. Such implication of glutamate is also documented in other neurological disorders and microdialysis studies have evidenced elevated levels of extracellular glutamate in and around glial tumours in both patients and experimental animal models of glioma (Roslin et al., 2003; Ye and Sontheimer, 1999; Behrens et al., 2000). Likely contributing to this excess of glutamate, reduced expression of glial glutamate transporters is a common feature of several human glioblastoma specimens and glioma cell lines (Ye et al., 1999). Notably, an inverse relationship between the expression of the most abundant glutamate transporter, the excitatory amino acid transporter 2 (EAAT2), and the tumour grade was recently confirmed (de Groot et al., 2005). Consistently, U87 glioma cells infected with an adenovirus coding for EAAT2 failed to generate tumours after subcutaneous implantation.

In addition to exhibiting noticeable deficits in typical astrocytic glutamate uptake activity, glioma cell lines and human glioblastoma have been shown to secrete substantial amounts of glutamate (Ye and Sontheimer, 1999). Similarly, centrally grafted C6 glioma cells were shown to release glutamate in the brain of Wistar rats (Takano et al., 2001) and the progression of tumour growth was correlated with the release capacity of selected cell clones. Hence, based on experimental observations in animal models, several authors have emphasised on the importance of glutamate release in the context of tumour growth. This spontaneous secretion of glutamate which largely depends on de-novo synthesis of glutamate from glutamine, has been correlated with the abundant expression of the glutamate-cystine exchanger (X_C^- antiporter) (Sato et al., 1999; Ye et al., 1999; Ye and Sontheimer, 1999).

In light of these observations, we generated C6 glioma cells in which expression of the glutamate transporter 1 (GLT-1, the rodent

Abbreviations: C6-rtTA, transfected C6 cells expressing rtTA; C6-rtTA-eGFP, transfected C6 cells with inducible expression of eGFP; C6-rtTA-GLT-1, transfected C6 cells with inducible expression of GLT-1; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride hydrate; DHK, dihydrokainic acid; EAAC1, excitatory amino acid carrier 1; EAAT2, excitatory amino acid transporter 2; eGFP, enhanced-green fluorescent protein; GLAST, glutamate and aspartate transporter; GLT-1, glutamate transporter 1; LTHA, L-(−)-threo-3-hydroxyaspartic acid; NMDA, *N*-methyl-D-aspartate; rtTA, reverse Tet-responsive transcriptional activator; X_C^- antiporter, glutamate/cystine exchanger.

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equivalent of EAAT2) could be manipulated in vivo, during tumour progression. Using this cellular model, we previously validated that enhancing glutamate transport activity of GLT-1 in C6 cells significantly reduced extracellular glutamate concentrations and significantly decreased cell proliferation in vitro (Vanhoutte and Hermans, 2008). These data suggested that glutamate released by the X_C^- antiporter is rapidly cleared from the medium through efficient reuptake by GLT-1, decreasing glutamate-induced proliferation of C6 glioma cells. The present study aimed at examining the hypothesis that enhancing GLT-1 activity in gliomas could interfere with tumour expansion in vivo by limiting glutamate-stimulated cell proliferation. In this purpose, this clone of inducible C6 cells was implanted in the striatum of adult rats. After validating the presence of a tumour, the expression of the transporter was induced and both tumour progression and animal survival were characterised.

Materials and methods

Cell culture

C6 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum and 50 μg/mL penicillin–streptomycin. All culture reagents were purchased from Invitrogen (Merelbeke, Belgium). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Transfection and cell selection

The establishment of an inducible expression system for GLT-1 or enhanced-green fluorescent protein (eGFP) in C6 glioma cells was previously described (Vanhoutte and Hermans, 2008). Briefly, a clone of C6 cells expressing the reverse Tet-responsive transcriptional activator (rtTA) was transfected with the pTet-On vector (Westburg, Leusden, The Netherlands) in which a 741 bp DNA fragment encoding the eGFP or a 1719 bp DNA fragment encoding the rat GLT-1B were cloned under the control of the doxycycline inducible promoter (respectively, C6-rtTA-eGFP and C6-rtTA-GLT-1).

Grafts and evaluation of tumour growth

Female Wistar rats were anaesthetised by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). For subcutaneous graft, 7×10^6 viable C6 cells in 100 μL of serum-free medium were implanted in the posterior paw of 8-week-old animals. Animals were observed daily for noticeable signs of tumour growth. When tumours were visible, doxycycline (500 μg/mL) was added to the drinking water and tumour size was recorded every 2–3 days. Tumour volume was measured using a digital calliper and calculated for each tumour using the formula: length × (width)^2 × 0.5 (Liu et al., 1999). For intrastriatal grafts, 5×10^4 viable C6 cells in 10 μL serum-free medium containing 1% agarose were injected using an Hamilton syringe through a burr hole in the right striatum (3 mm lateral, 0 mm anterior to the bregma point, 5 mm deep from the dura) of animals (250–300 g) immobilised in a stereotactic frame (David Kopf Instruments, Tujunga, USA) (Kobayashi et al., 1980).

In order to evaluate tumour progression, an animal group was sacrificed 25 days after graft. These rats were anaesthetised by an intraperitoneal injection of ketamine/xylazine and perfused intracardially with physiological saline solution followed by buffered-paraformaldehyde 4%. Brains were isolated and fixed for an additional 48 h, embedded in paraffin, and sectioned using a microtome (Microm HM 340E, Microm International, Walldorf, Germany). Serial sections of 5 μm were stained with hematoxylin/eosin and scanned using a camera coupled to an imaging station allowing to estimate tumour size (MCID M4, Linton, UK). Tumour volume was calculated for each animal using the formula: 4/3 π × largest area × number of sections × thickness of sections. All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC).

Magnetic resonance imaging (MRI)

High resolution MRI of animal brain was performed every week after graft of the cells. After anaesthesia with isoflurane (3% induction, 1.8% maintenance), rats were imaged in a 4.7 T (200 MHz, 1 H) magnet with a 40 cm inner diameter bore system (Bruker Biospec, Ettlingen, Germany). A birdcage radiofrequency (RF) coil with an inner diameter of 70 mm was used. Animal temperature was maintained by flushing warm air inside the magnet. Anatomical anatomical T2-weighted images were acquired using a fast spin echo sequence (RARE or Rapid Acquisition with Relaxation Enhancement), with a repetition time (TR), effective echo time (TE) and echo train length of 4162.1 ms, 50.5 ms (N averages = 16; matrix size = 128 × 128, and total acquisition time = 17 min 27 s) (Maron et al., 1996). Series of six contiguous axial slices of 1 mm were acquired, with a spatial resolution of 273 μm by pixel. The tumour size was evaluated by hand-drawing regions of interest (ROIs) in the median tumour slice using the MATLAB software. Tumour ROIs were then reported to those measured for the brain on the same slice.

eGFP fluorescence analysis, immunocytochemistry and immunohistochemistry

For immunocytochemistry, cells were grown on coated 12 mm round glass coverslips. For immunohistochemistry, brains were removed and immediately frozen in 2-methylbutane cooled with liquid nitrogen and sectioned to 10 μm using a freeze microtome (Microm HM 500, Microm International). The preparations were then fixed with paraformaldehyde 4% (v/v) for 30 min or acetone for 2 min at room temperature and permeabilised with 1% Triton X-100 (v/v) in PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) during 15 min. Non-specific binding was blocked by incubating the cells in a PBS solution containing normal donkey serum 10% (Jackson Immunoresearch, DePinte, Belgium) for 20 min at 37 °C. Samples were then incubated overnight at 4 °C with primary antibodies, i.e. a goat antibody raised against the N-terminus of EAAT2 (1:500, Santa Cruz Biotechnology, California, USA), a mouse anti-rtTA antibody (1:500, Westburg, Leusden, The Netherlands) and a rabbit anti-MAP2 antibody (1:1000, a gift from Jean-Pierre Brion, Université Libre de Bruxelles, Brussels, Belgium). Secondary antibodies, applied for 1 h at room temperature, were FITC-conjugated donkey anti-goat IgG antibody (1:500, Chemicon, Hampshire, UK), FITC-conjugated donkey anti-rabbit IgG antibody (1:500, Chemicon), Cy3-conjugated donkey anti-mouse IgG antibody (1:500, Chemicon) and Cy3-conjugated donkey anti-rabbit (1:500, Jackson Immunoresearch Laboratory, DePinte, Belgium). Nuclei were stained during 30 min with the nuclear dye DAPI (1:5000, Sigma-Aldrich, Bornem, Belgium). After three rinses in PBS, the preparations were mounted in Fluoprep (BioMerieux, Brussels, Belgium). Preparations were examined on an Olympus IX70 inverted fluorescent microscope coupled to a CCD camera (TILL Photonics, Martinsried, Germany). Excitation light (475, 488, 540 and 400 nm for eGFP, FITC, Cy3 and DAPI, respectively) was obtained from a Xenon lamp coupled to a monochromator (T.I.L.L. photonics). Digital images were acquired and analysed using the TILLvisION software (T.I.L.L. photonics). For ex vivo fluorescence analysis, animals were sacrificed and the removed brains were examined on a digital imaging system (IS4000MM, Eastman Kodak Company, Rochester, NY, USA) coupled to a CCD camera and excitation filter (465 nm). Digital images were acquired and analysed using the Kodak 1D Image Analysis Software.

Measurement of D-[^{3}H]-aspartate transport activity in glioma synaptosomes

Synaptosomes were prepared as previously described by Bonnet and Costentin (1989), with minor modifications. Surgically removed striatum or glioma tissues were homogenised in 20 volumes of 0.32 M sucrose using 10 up-and-down strokes of a pre-chilled Teflon-glass homogeniser. The homogenates were then centrifuged at 1000 g for 10 min at 4 °C. Supernatants were stored at 4 °C and the pellet was resuspended in 20 volumes of 0.32 M sucrose and centrifuged again for 10 min at 1000 g. The two supernatants were then pooled and centrifuged at 17,500 g for 30 min at 4 °C, after which the supernatant was discarded and the final pellet resuspended in ice-cold Krebs-Ringer buffer (120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 6 mM glucose, pH 7.6). Glutamate transport was assayed using D-[^{3}H]-aspartate (Perkin-Elmer NEN, Belgium) as substrate, at a tracer concentration of 50 nM. Synaptosomes prepared from 10 µg of tissue were incubated in 500 µL of Krebs-Ringer buffer with D-[^{3}H]-aspartate for 10 min at 37 °C. Non-specific transport was measured in the presence of inhibitor L-(−)-threo-3-hydroxyaspartic acid (LTHA, 100 µM, Tocris, Bristol, United Kingdom) and GLT-1-sensitive transport evaluated using dihydrokainic acid (DHK, 100 µM, Tocris). Three millilitres ice-cold Krebs-Ringer buffer were added and aspartate transport was stopped by immediately filtering the suspension under vacuum through Whatman GF/B filters. Vials and filters were washed twice with 3 mL of ice-cold Krebs-Ringer buffer. The filters were placed in scintillation vials containing 7 mL of Aqualuma (Lumac, Groningen, The Netherlands) before being stored at room temperature overnight. Radioactivity was determined by liquid scintillation spectrometry. Protein content was measured by the method of Peterson (1977) using bovine serum albumin as standard and D-[^{3}H]-aspartate transport was expressed as pmol/mg protein/min.

Results

Induction of GLT-1 expression in C6 cells limits tumour growth after subcutaneous graft

We previously reported on the use of a tetracycline-driven inducible system in order to generate stably transfected C6 cells in which expression of the glutamate transporter GLT-1 was modulated by exposing the cells to doxycycline. Immunocytochemical studies showed that exposure of these cells to doxycycline (2 µM) for 72 h robustly increased the expression of GLT-1, an effect that was correlated with enhanced substrate uptake capacity (see Vanhoutte and Hermans (2008) and Figs. 3A and B). A preliminary evaluation of the putative effect of GLT-1 expression on in vivo tumour progression was obtained by subcutaneous graft of these cells into the posterior paw of rats. Seven days after cell implantation, doxycycline (500 µg/mL) was added to the drinking water and tumour size was measured every other days. Doxycycline treatment was found to rapidly impede tumour growth, resulting in significantly smaller tumour volume as compared to vehicle-treated rats ($p = 0.011$, paired non-parametric test, $n = 8$ in each group) (Figs. 1A and C). When the same experiment was conducted with transfected C6 cells lacking the coding sequence of GLT-1 (C6-rtTA), the mean volume of tumours measured in animals treated with doxycycline was not significantly different from that measured in vehicle-treated animals ($p = 0.320$, paired non-parametric test, $n = 8$ in each group) (Fig. 1B). It is noteworthy that in all cases, the tumour spontaneously regressed in these models of subcutaneous grafts.

Efficient inducible expression of eGFP or GLT-1 in C6 cells implanted in the rat striatum

A model of transfected C6 cells expressing eGFP under the control of the inducible promoter (C6-rtTA-eGFP cells) was used in order to

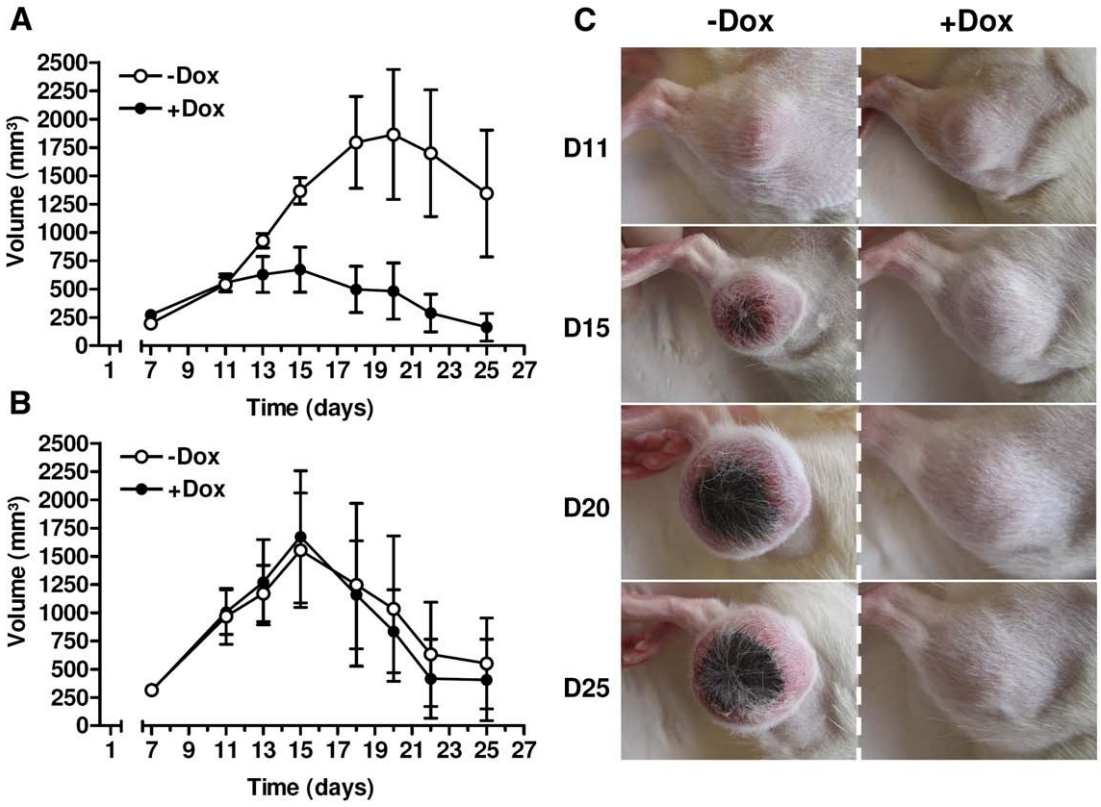

Fig. 1. Influence of GLT-1 expression on the growth of C6 glioma cells in a model of subcutaneous grafts. Volumes of tumours developing after subcutaneous implantation of C6-rtTA-GLT-1 cells (A) and C6-rtTA cells (B) in the paw of Wistar rats, treated or not with doxycycline (500 µg/mL in drinking water). Data shown are means ± S.E.M. ($n = 8$ animals in each group). (C) Images of subcutaneous tumours obtained with C6-rtTA-GLT-1 cells at days 11, 15, 20, 25 in animals treated or not with doxycycline.

optimise doxycycline activation of the expression system in the rat brain. In vitro, the addition of doxycycline (2 μM) to the culture medium increased the emission of green fluorescence by these cells after 72 h (Fig. 2A). In contrast, fluorescence was barely detected in non-induced cells, validating the efficiency of the controlled expression system (Fig. 2B). Rats injected with these cells in the striatum by stereotaxy developed tumours that reached approximately 6 mm diameter, 25 days after implantation. The addition of doxycycline to the drinking water (500 μg/mL) for the last 4 days caused a robust increase in the expression of eGFP at the site of the tumour, as evidenced by fluorescence imaging on the entire brain removed from the skull (Fig. 2C). Even though a doxycycline concentration of 2 mg/mL in the drinking water is commonly used (which corresponds to 100 mg/kg in rats) (Dhawan et al., 1995), our initial assays revealed equivalent inductions of eGFP after treatments with 2 mg/mL and 500 μg/mL. Avoiding potential side effects during prolonged treatments, the lowest concentration was used in all further experiments. As expected, no signal was associated with the tumours of rats receiving vehicle (not shown).

These implantations were repeated with the clone of C6 cells carrying the GLT-1 construct. Twenty five days after receiving C6-rtTA-GLT-1 cells in their right striatum, rats were sacrificed, brains were removed and immunohistochemical analyses were conducted on frozen tissue sections. Tumour tissue was easily distinguished from brain parenchyma by the high density of cell nuclei. In addition, the tumour mass could be distinguished from host brain structures by immunodetection of the rtTA which is specifically expressed in the transfected C6 cells (Figs. 3C and D). It is noteworthy that the vast majority of cells in the tumour stained positive for rtTA, revealing that the tumour was mainly composed of C6 cells. Accordingly, the tumour was also characterised by an almost total absence of neuronal cells, detected by MAP2 immunolabelling (Fig. 3D). Indeed, the perimeter of the tumour was clearly identified and only a few C6 cells were found to invade the brain parenchyma. Consistent with our previous in vitro characterisation of C6-rtTA-GLT-1 cells revealing that the inducible system was not totally silent in the absence of doxycycline (Vanhoutte and Hermans, 2008), a basal expression of GLT-1 in tumour cells was evidenced in animals that did not receive doxycycline. However, in rats treated for 4 consecutive days with doxycycline before sacrifice, the expression of GLT-1 was markedly enhanced in the vast majority of tumour cells but not in the adjacent endogenous cells (Figs. 3E–F).

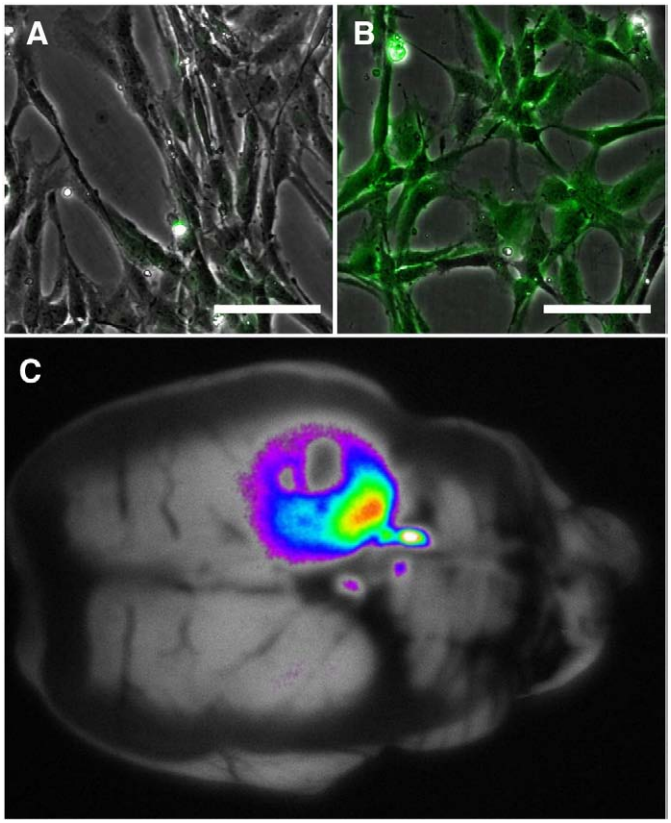

Fig. 2. Doxycycline-inducible expression of eGFP in C6 glioma cells. (A and B) respectively show detection of eGFP in cultured C6-rtTA-eGFP cells exposed or not to 2 μM doxycycline for 72 h. Fluorescence and phase contrast transmission images were combined. (C) shows the fluorescent signal detected in the brain of a rat developing a tumour 25 days after cerebral implantation of C6-rtTA-eGFP cells and having received doxycycline (500 μg/mL in drinking water) during the last 4 days. Intensity of fluorescence is indicated using a standard rainbow colour scale. Scale bar = 50 μm.

Enhanced expression of GLT-1 reinforces aspartate uptake in C6 cells grafted into the rat striatum

The glutamate transporter activity in the tumours was evaluated by measuring the D-[^{3}H]-aspartate (50 nM) uptake in synaptosomal suspensions prepared from surgically removed tissues. For these functional uptake studies, the protocol for doxycycline induction was specifically adapted. Thus, as the aim was to confirm the in vivo induction of GLT-1 activity by doxycycline, series of grafted rats were left untreated for 21 days before starting the induction for a short period of 4 days by adding (or not) doxycycline in the drinking water. This procedure was required in order to obtain large sized tumours in both induced and non-induced animals, facilitating their dissection in the absence of contaminating brain tissue. Confirming the deficit in glutamate transporter activity commonly observed in gliomas, the experiments conducted on samples from vehicle-treated rats (no doxycycline) revealed a modest but significant aspartate uptake. As shown in Fig. 4, this uptake was lower as compared to the values measured in synaptosomes from striatum of non-grafted animals (respectively, 3.19 ± 0.27 pmol min^{-1} mg prot^{-1} and 10.03 ± 0.64 pmol min^{-1} mg prot^{-1}, samples from 3 rats in each group). The aspartate uptake in synaptosomes prepared from tumour samples was totally inhibited by LTHA, a non-selective inhibitor of glutamate transporters, but was not significantly modified by the selective GLT-1 blocker DHK. In contrast, the uptake measured in striatal tissues from non-grafted animals was partially inhibited by DHK (25%) confirming the activity of GLT-1 in the healthy adult brain. Consistent with the inducible expression of GLT-1 in C6-rtTA-GLT-1 cells grafted rats, the administration of doxycycline to these animals resulted in a robust reinforcement of the substrate transport velocity in synaptosomes prepared from their tumours (176% increase as compared to samples from vehicle-treated rats, $p<0.001$, one-way ANOVA followed by Newman–Keuls test, samples from 3 rats in each group). This enhanced uptake was almost completely absent when tested in the presence of DHK demonstrating the specific reinforcement of GLT-1 activity (no significant difference between samples from doxycycline-treated compared to vehicle-treated rats both in the presence of DHK, $p>0.05$, one-way ANOVA followed by Newman–Keuls test). As expected, the administration of doxycycline was without influence on the substrate uptake measured in samples from non-grafted animals or in tumours obtained after implantation of C6-rtTA cells, which lack the GLT-1 transgene (not shown).

Enhancing GLT-1 expression in grafted C6 cells decreases glioma progression

In order to evaluate the influence of GLT-1 induction on the growth of glioma, C6-rtTA-GLT-1 cells were grafted in the right striatum of rats and the tumour progression was monitored by MRI. Seven days after cells implantation, as the presence of a growing tumour mass was confirmed, doxycycline was added to the drinking water of half of the animals ($n=4$ in each group). At that stage, tumours were

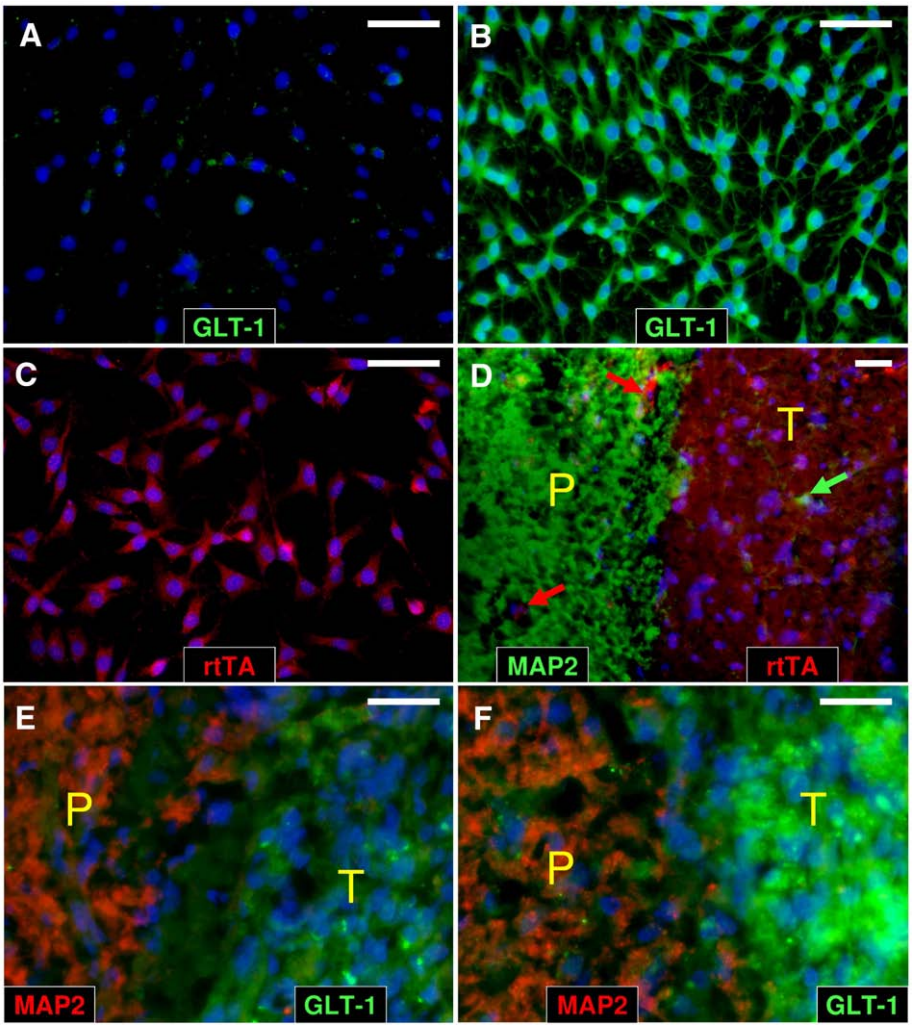

Fig. 3. Inducible expression of GLT-1 transporter in transfected C6 glioma cells. The expression of the transactivator rtTA and the GLT-1 glutamate transporter in C6-rtTA-GLT-1 cells was examined in vitro (A–C) and in vivo after intrastriatal implantation (D–F) using specific antibodies. The exposure of C6-rtTA-GLT-1 cell cultures to doxycycline (2 μM) for 72 h dramatically increased the expression of GLT-1 (B) in comparison to non-exposed cells (A). The specific expression of rtTA which was validated in cultures (C) was used to trace the C6 cells after implantation into the brain (D). Merging the immunostaining for rtTA and MAP2 revealed the presence of few tumour cells invading the nervous tissue (red arrows) as well as limited infiltration of neurons in the tumour (green arrow) (D). Merging the immunostaining for rtTA and GLT-1 indicated that in comparison to controls (E), the administration of doxycycline (500 μg/mL in drinking water) for 4 days robustly increased the expression of GLT-1 in the tumour but not in the surrounding nervous tissue (F). GLT-1 immunoreactivity was detected with a FITC-coupled secondary antibody (green), rtTA immunoreactivity was detected with a Cy3-coupled secondary antibody (red) and MAP2 immunoreactivity was detected with FITC-coupled secondary antibody (green) in image D and a Cy3-coupled secondary antibody (red) in images E and F. Cell nuclei were stained with DAPI (blue). Scale bar = 50 μm. Data shown are representative of experiments performed on 3 rats in each group.

observable by MRI as a dense crown surrounding a dark nucleus corresponding to agarose injected with the cells (Fig. 5A). In vivo brain imaging was conducted during 3 weeks and revealed that the tumours expanded slower in the group of animals receiving doxycycline (Figs. 5A and B). To better characterise the effect of doxycycline on tumour growth, several animals were sacrificed after 25 days and brains were removed in order to evaluate tumour volume on eosin-hematoxylin stained sections (Fig. 6A). At this stage, the mean volume of C6-rtTA-GLT-1 tumours was significantly lower in animals treated with doxycycline as compared to controls (18.85 ± 5.20 mm^3 and 122.54 ± 32 mm^3, respectively; $p<0.05$, one-way ANOVA followed by Newman–Keuls test, $n=4$ in each group) (Fig. 6B). Similar experiments conducted with animals injected with C6-rtTA cells revealed that doxycycline was without significant influence on the mean volume of tumours lacking inducible expression of GLT-1 (167.35 ± 44.23 mm^3 and 178.17 ± 61.36 mm^3, respectively; $p>0.05$, one-way ANOVA followed by Newman–Keuls test, $n=4$ in each group).

Finally, the consequence of inducing GLT-1 expression and activity in implanted C6 cells on animal survival was examined. As illustrated in Fig. 6C, animals carrying a tumour derived from C6-rtTA-GLT-1 cells survived significantly longer when they were treated with doxycycline as from day 7 after graft ($p=0.006$, log rank test, minimum of 8 rats in each group). The mean survival time was 47.6 ± 2.4 and 37.3 ± 2.0 days for doxycycline and vehicle-treated animals, respectively. Worth mentioning, 2 rats injected with C6-rtTA-GLT-1 cells and treated with doxycycline were still alive at day 70 after implantation and no tumour was visible after autopsy of their brains. These animals were not included in the analysis of animal survival time. Finally, no significant difference was observed in the survival time of doxycycline- and vehicle-treated animals carrying C6-rtTA implants (respectively 33.5 ± 3.0 and 31.2 ± 2.2 days, $p=0.203$, log rank test, minimum of 6 rats in each group) (Fig. 6C).

Discussion

In this study, we have shown that the manipulation of glutamate uptake in intrastriatally implanted glioma cells influences the tumour growth and the survival time of animals. The use of a tetracycline-

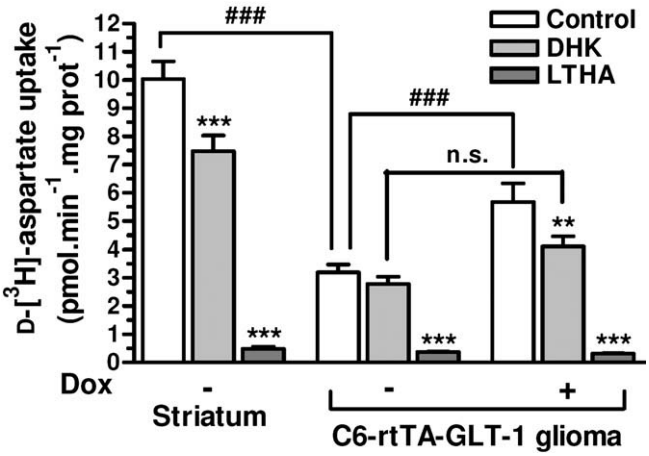

Fig. 4. Enhanced glutamate transporter activity in C6-rtTA-GLT-1-derived tumours in animals treated with doxycycline. Functional assays were conducted on synaptosomes prepared from surgically removed gliomas. For these functional assays, C6-rtTA-GLT-1 cells were grafted and animals were left untreated for 3 weeks, allowing the development of large sized tumours. Thereafter, some animals received doxycycline (500 μg/mL in drinking water) for 4 days. Compared to striatum samples from non-grafted animals, gliomas derived from C6-rtTA-GLT-1 cells showed a modest but significant D-[^{3}H]-aspartate uptake when animal were not treated with doxycycline. In contrast, administration of doxycycline significantly increased the uptake in these gliomas. The specificity of the uptake was examined using LTHA (100 μM) whereas the involvement of GLT-1 was evaluated using DHK (100 μM). Data shown (means with S.E.M.) are from experiments conducted on 3 rats in each group in triplicate. n.s. = non significant, $^{**}p<0.01$, $^{***}p<0.001$, $^{\#\#\#}p<0.001$ (one-way ANOVA followed by Newman–Keuls test).

dependent expression system combined with the choice of doxycycline which efficiently crosses the blood–brain barrier allowed us to specifically induce the expression of the high affinity glutamate transporter GLT-1 several days after implantation of the C6 cells. The robust induction of the glutamate transporter expression, which was initially demonstrated in vitro, was validated in vivo by showing positive GLT-1 immunoreactivity in the grafted cells, exclusively after doxycycline administration. Indeed, the GLT-1 immunostaining in the tumour cells was higher as compared to the signal detected in the proximal brain parenchyma. Functional assays conducted on synaptosomes prepared from surgically collected tumours confirmed that the treatment of animals with doxycycline enhanced the aspartate uptake activity depending on GLT-1. Of note, uptake values measured in these tumour samples was not as high as expected from the in vitro characterisation of the transfected cells. This observation raises questions regarding the actual concentration of doxycycline that efficiently reaches the glioma cells in growing tumours, where vascularisation is likely to be disorganized. Alternatively, such discrepancy may also result from methodological differences in the functional assays conducted either on intact cultured cells or on tumour synaptosomes.

Volumetric analysis revealed that the expansion of the tumour in animals treated with doxycycline was delayed and this effect correlated with extended survival of rats carrying these tumours, as compared to animals treated with the vehicle. Even though one could speculate on the mechanisms involved in the influence of GLT-1 and glutamate on tumour progression, it remains difficult to isolate a single process. Accumulating reports indicate that glutamate released by glioma cells promotes cell proliferation and motility via autocrine and/or paracrine activation of glutamate receptors. The trophic effects of glutamate in glioma cells were notably assigned to the activation of ionotropic or metabotropic glutamate receptors as their pharmacological blockade was shown to limit glioma growth, both in vitro and in vivo (Arcella et al., 2005; D'Onofrio et al., 2003; Ishiuchi et al., 2002; Rzeski et al., 2001; Takano et al., 2001). Furthermore, genetically modified U251 and U87 glioma cells lacking GluR1 (GluA1), the most abundant ionotropic glutamate receptor subunit in glioma, showed a reduced growth capacity in a mouse model of subcutaneous graft (de Groot et al., in press). In accordance with this hypothesis, we previously showed that the exposure to glutamate increased the proliferation of cultured C6 cells and that inducing GLT-1 activity in these cells, which significantly reduced glutamate concentrations in the culture medium, resulted in a decreased cell proliferation rate (Vanhoutte and Hermans, 2008). These data suggested that the restoration of an efficient uptake activity in C6 cells may counterbalance their intrinsic capacity to release glutamate, thereby limiting its influence on cell proliferation. The possible influence of extracellular glutamate on C6 cell proliferation is corroborated by experiments involving the graft of the glioma cells in peripheral tissues. Indeed, the progression of subcutaneous tumours derived from C6-rtTA-GLT-1 cells was strongly impaired in animal receiving doxycycline. Even though glutamate could also serve as a cell nutriment after intracellular metabolism, these data also exclude that promoting its uptake would favour cell proliferation through enhanced metabolism fuelling.

Obviously, beside an influence on tumour cell proliferation, glutamate released by glioma is also known to facilitate tumour growth by triggering excitotoxic lesions in the peritumoural nervous

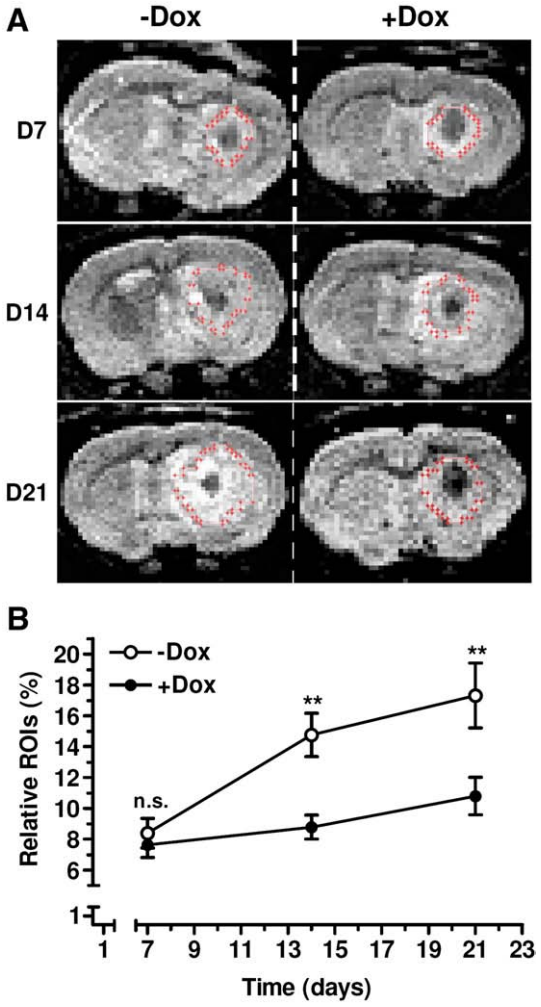

Fig. 5. Influence of induced GLT-1 expression in C6 cells on tumour progression in vivo. (A) Images obtained by MRI showing the progression of C6-rtTA-GLT-1 glioma at days 7, 14 and 21 after graft. Those are representative images from studies conducted with 4 rats in each group. Tumour regions of interest are delimited by a hand drawn red line on each slice (see Materials and methods). (B) Measures of relative size of the tumour (tumour/total brain areas ratio) indicate that tumours show slower expansion when animals were treated with doxycycline (500 μg/mL in drinking water), as compared to untreated animals.

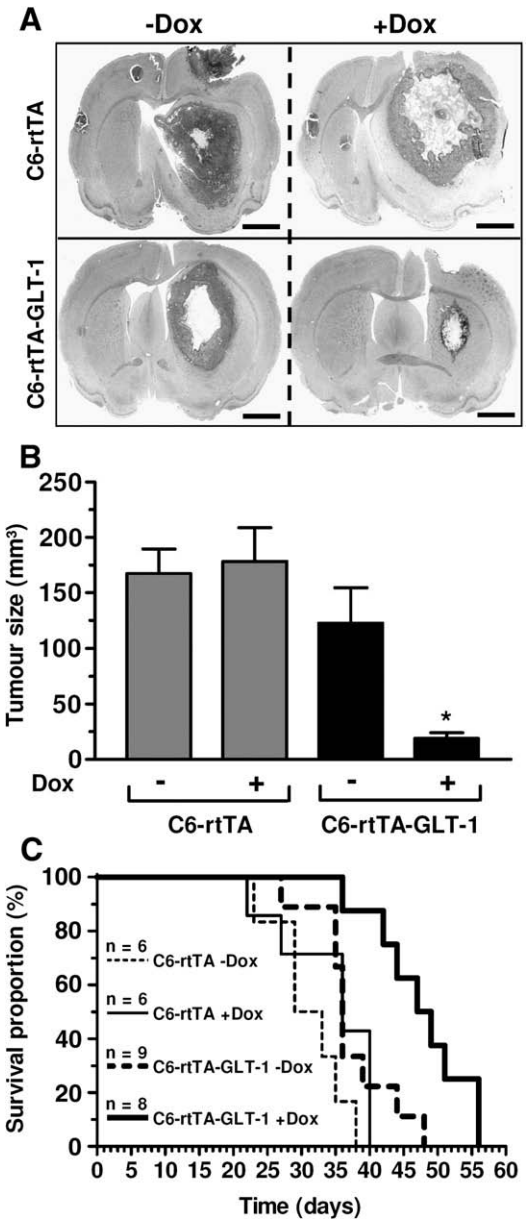

Fig. 6. Enhanced expression of GLT-1 in C6 glioma cells decreases tumour progression. Brain sections were prepared from animals sacrificed 25 days after implantation of C6-rtTA or C6-rtTA-GLT-1 cells and analysed after staining with hematoxylin/eosin (A). These animals have been exposed or not to doxycycline (500 μg/mL in drinking water) since day 7 after implantation. Scale bar = 3 mm. Tumour volumes were measured on 4 rats in each group, 25 days after implantation (B). Data shown are means with S.E.M. ($n = 4$). *$p < 0.05$ (one-way ANOVA followed by Newman–Keuls test). Other cohorts of animals bearing C6-rtTA or C6-rtTA-GLT-1 derived tumours and treated or not with doxycycline were kept alive in order to determine their survival time, illustrated here in a Kaplan–Meier survival plot (C). The number of rats used for the survival experiments are indicated on the graph.

tissue (Takano et al., 2001; Ye and Sontheimer, 1999). Therefore, one may hypothesise that enhancing GLT-1 expression and activity in C6 glioma cells implanted in the brain would interferes with the local glutamate-mediated neuronal insult. Indeed, apoptosis and neuronal cell death were previously evidenced in regions surrounding C6 gliomas and the role of glutamate was clearly demonstrated (Takano et al., 2001). This concept has been largely developed and the involvement of glutamate-induced neurotoxicity in the peritumoural region during tumour expansion was clearly established. Indeed, Takano and colleagues notably showed the potential benefit brought by antagonists of neuronal *N*-methyl-D-aspartate (NMDA) receptors (memantine or MK-801) on the progression of gliomas. While the present study did not aim at demonstrating the relative importance of these different mechanisms in the tumour progression, we herein clearly demonstrate that enhancing the uptake of glutamate by the glioma cells (or at the vicinity of the tumours) could present an effective therapeutic approach. Worth mentioning, the administration of small molecule compounds promoting GLT-1 upregulation was recently shown to prevent tumour growth in an animal model of experimental glioma (Coddington et al., 2008).

On the basis of the impressive decrease in tumour size detected 25 days after graft, one would expect that the survival of the animals treated with doxycycline would be much more extended than observed. It is likely that glioma growth is accompanied by changes in the local blood perfusion of the tumour and that the doxycycline supply to deep layers of the tumour might be compromised at late stages. In addition, as the tumour progresses the massive destruction of the blood–brain barrier could facilitate the entry of high concentrations of glutamate in the brain tissue, overcoming the capacity of clearance achieved by the recombinant glutamate transporter. This could explain why the benefit on tumour growth would progressively fade leading to an accelerated process and an eventual death.

These limitations are indeed related to the nature of inducible expression system used in this study. Nevertheless, this model has allowed to examine two groups of rats implanted with the same cell clone, avoiding the comparison of rats carrying transfected and non-transfected cells that would inevitably constitute an experimental bias. Indeed, some preliminary experiments revealed intrinsic differences in the growth of tumours generated with C6-rtTA and C6-rtTA-GLT-1 cells, in animals not exposed to doxycycline. Besides, using either C6-rtTA or C6-rtTA-GLT-1 cells, the tumours spontaneously regressed in the model of subcutaneous grafting, an effect that is not observed in the model of striatal implantation. This spontaneous regression was previously documented in the subcutaneous C6 cell model (Watanabe et al., 2002), and was assigned to the necrosis of the tumour following the immune rejection of this allogenic graft (Barth, 1998). Such regression is not observed after graft of the glioma cells in the brain, as in this case, the growth of the tumour is rapidly fatal. Noteworthy, excitotoxic insults in the central nervous system are frequently associated with inflammatory responses that may contribute to degenerative processes (Tilleux and Hermans, 2007). Indeed, increased inflammation is commonly observed at the proximity of brain tumours and several studies have highlighted the possibility to influence tumour progression using both pro- and anti-inflammatory approaches (Nakano et al., 2006). The anti-inflammatory properties of several tetracycline derivatives such as minocycline are well documented (Pasquale and Tan, 2005) and one could suspect that the benefits obtained with doxycycline involve a modulation of local inflammation. Nevertheless, administration of doxycycline to animals carrying a tumour derived from C6-rtTA cells produced neither a significant delay in tumour growth nor a substantial increase in survival time. It is worth noting that the peritumoural inflammation is accompanied by a diffuse edema that was clearly evidenced in MRI images. Indeed, histological analysis revealed that tumour sizes were overestimated as this non-invasive technique evidences both tumour cells and peritumoural edema. Even though no toxicological evaluation has been conducted, the animals have been observed on a daily basis, and no obvious sign of discomfort or unwanted effects have been recorded during the doxycycline treatment. Indeed, previous studies have validated the lack of severe toxicity of doxycycline in rats at the dose used (Dietz et al., 1991). Hence, if doxycycline was harmful for the animals, one could predict that the enhanced lifespan in animal carrying tumours where GLT-1 is induced has been underestimated.

In summary, we herein provide evidence that promoting the glutamate uptake capacity in C6 cells decreases their growth when implanted subcutaneously or in the central nervous system. These

observations indirectly support the hypothesis that an intrinsic deficiency in glutamate transporter expression commonly observed in glioma cells is a key pathogenic feature of these glial tumours. This adds these malignant gliomas to the list of neurological disorders in which an excess of extracellular glutamate contributes to the progression of the disease. Moreover, these data also highlight the relevance of developing pharmacological approaches aiming at ensuring a better control of extracellular glutamate concentrations to reduce the invasive behaviour of glioma.

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